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Multi-institutional oncogenic driver mutation analysis in lung adenocarcinoma: The Lung Cancer Mutation Consortium experience

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Abstract

Introduction—Molecular genetic analyses of lung adenocarcinoma have recently become standard of care for treatment selection. The Lung Cancer Mutation Consortium was formed to enable collaborative multi-institutional analyses of 10 potential oncogenic driver mutations. Technical aspects of testing, and clinicopathologic correlations are presented.

Methods—Mutation testing in at least one of 8 genes (*EGFR, KRAS, ERBB2, AKT1, BRAF, MEK1, NRAS, PIK3CA)* using SNaPshot, mass spectrometry, Sanger sequencing +/− PNA and/or sizing assays, along with *ALK* and/or *MET* FISH were performed in 6 labs on 1007 patients from 14 institutions.

Results—1007 specimens had mutation analysis performed, and 733 specimens had all 10 genes analyzed. Mutation identification rates did not vary by analytic method. Biopsy and cytology specimens were inadequate for testing in 26% and 35% of cases compared to 5% of surgical specimens. Among the 1007 cases with mutation analysis performed, *EGFR, KRAS, ALK*, and *ERBB2* alterations were detected in 22, 25, 8.5, and 2.4% of cases, respectively. *EGFR* mutations were highly associated with female sex, Asian race, and never smoking status; and less strongly associated with stage IV disease, presence of bone metastases, and absence of adrenal metastases. *ALK* rearrangements were strongly associated with never smoking status, and more weakly associated with presence of liver metastases. *ERBB2* mutations were strongly associated with Asian race and never smoking status. Two mutations were seen in 2.7% of samples, all but one of which involved one or more of *PIK3CA, ALK* or *MET*.

Conclusion—Multi-institutional molecular analysis across multiple platforms, sample types, and institutions can yield consistent results and novel clinicopathological observations.

Keywords

lung adenocarcinoma; mutation; FISH; genotyping; LCMC

INTRODUCTION

Molecular genetic testing is a central component of pathological analysis for several types of cancers. Although results formally reported in the medical record must be generated in laboratories subject to Clinical Laboratory Improvement Amendments (CLIA) certification, the degree of inter-laboratory variation with regard to molecular pathology methods and results remains poorly characterized outside of laboratory proficiency testing and studies designed specifically to evaluate concordance. Molecular testing in patients with advanced lung cancer and other solid tumors presents unique challenges. The use of minimally invasive procedures to obtain tissue for diagnosis often limits available tumor material for molecular testing. Variable pre-analytic methodology introduces the potential for poor nucleic acid preservation in formalin-fixed, paraffin-embedded (FFPE) tumor samples. Furthermore, the continuing identification of new driver mutations can lead to repetitive testing of the same sample exhausting the material available.

Molecular genetic testing became central to the clinical management of advanced lung adenocarcinoma (ACA) after the discovery of a strong association between activating *EGFR*

mutations and clinical response to targeted EGFR tyrosine kinase inhibitors (TKIs) in 2004 [1-3]. This was followed by the identification of rearrangements of the anaplastic lymphoma kinase (*ALK*) gene in lung ACA in 2007, which are in turn uniquely sensitive to treatment with ALK kinase inhibitors [4,5]. *EGFR* mutation analysis and *ALK* fluorescence in situ hybridization (FISH) are now guideline-recommended standard-of-care at the time of diagnosis for advanced lung ACA to inform the initial systemic treatment [6]. Ongoing recognition of potentially targetable oncogenic drivers in lung ACA [7] indicates a need for efficient multiplexed analyses. Indeed, many institutions in the US and worldwide have implemented routine analyses of multiple genes in lung ACA [8-10]. A growing number of commercial and academic institutions are implementing next generation sequencing (NGS) of large gene panels as a more efficient approach to molecular testing across multiple cancer types [11,12, 13].

The Lung Cancer Mutation Consortium (LCMC) was established in 2008 as a multi-institutional program investigating the frequency of selected oncogenic drivers in lung ACA and using the results to treat the enrolled subjects with targeted therapies, either as part of standard clinical care or on investigational protocols. Fourteen institutions participated in the LCMC and either performed testing locally or utilized another LCMC site. Analytical methods at testing sites were left up to at each institution, as long as they met CLIA standards.

The primary results of the LCMC study have recently been reported. [14] Here we provide additional information on methods used at the different institutions, results of blinded proficiency testing, effects of sample type and testing platform on assay success and mutation detection rates, and validation of mutations identified in lung cancer specimens with more than one putative driver alteration. Further, we examine sample failure rates and present a correlation between the presence of oncogenic driver mutations and clinicopathologic findings.

MATERIALS AND METHODS

Patient Recruitment and Enrollment

Fourteen clinical sites participated in the LCMC (Supplemental Table 1). All participating sites obtained local IRB approval for participation in this study. Patients with stage IV or recurrent lung ACA; SWOG performance status of 0, 1, or 2; expected survival of >6 months; and adequate tissue for molecular analyses were eligible for entry on this study. 1,542 patients were enrolled, and 1,102 were deemed eligible. The most common reason for ineligibility was inadequate pathologic material to complete the multiplexed testing (n=286 of 440 ineligible; 65%). Epidemiologic and clinicopathologic data was collected on these subjects, including age, sex, race, smoking history, stage at diagnosis, metastatic sites, and survival. [14]

Pathology evaluation

Anatomic pathologists at each institution confirmed a diagnosis of lung adenocarcinoma, assessed tumor content, and determined specimen adequacy based upon analytic sensitivity

of their testing platform (Table 1). Samples were enriched for tumor content using manual microdissection. Central confirmation of lung ACA diagnosis was based on review of an hematoxylin and eosin (H&E) stained histology slide or a scanned image (Aperio®, Vista, CA), by IIW, JF, or WAF. At the time of central review, expert pathologists enumerated percentage of each histologic pattern, including lepidic, acinar, papillary, micropapillary, solid and variants (mucinous, colloid, fetal, and enteric, as appropriate), according to current criteria. [15]

Among the 1,102 eligible patients, 1,015 were confirmed as ACA histology and two as adenosquamous carcinoma by central pathology review. In 85 cases, slides were not provided for central review. Among cases with confirmed histology, at least one molecular assay was performed in 1,007 cases. Small mutations were defined as single nucleotide variants and small insertion-deletion (indel) mutations. Testing for at least one small-mutation gene (8 genes, see below) was performed for 989 cases, *ALK* FISH testing was performed in 926 cases, and *MET* FISH testing in 833. The 10-marker panel including small mutation and FISH testing was completed in its entirety for 733 patients.

Mutational Analyses

The vast majority of the mutation analyses were performed in six diagnostic laboratories, using methods summarized in Table 1. The complete panel of small mutations consisted of four small indels and 93 point mutations occurring in 8 genes (AKT1, BRAF, EGFR, ERBB2, KRAS, MAP2K1, NRAS, PIK3CA) (Supplemental Table 2). Due to variability in testing platforms, not all mutations were evaluated at all sites, but every site tested at least half of the complete set of mutations.

Three different methodologies were used for genotyping, and the analytic sensitivities for the major testing laboratories (defined as those testing ≥ 4% of the total cases) are shown in Table 1. The methods for all mutational analyses have been previously published [15-17]. Briefly, SNaPshot® (Life Technologies, Grand Island, NY) is a multiplex PCR assay followed by single-base primer extension. Sequenom MassARRAY (Sequenom, San Diego, CA) is a multiplex PCR assay followed by single base extension sequencing and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). PCR followed by sizing assay for *EGFR* exons 19 and 20 and *ERBB2* exon 20 using fluorescently-labeled primers and capillary electrophoresis was used to detect the indel mutations for both SNaPshot and Sequenom [17]. For bidirectional Sanger sequencing, assay sensitivity was enhanced using peptide nucleic acid clamps (PNAs) to suppress amplification of the wild-type allele at mutation hotspots in *EGFR*, *KRAS*, and *BRAF*. PNA clamp design for *EGFR* and *KRAS* has been previously described [18,19]; clamp design for *BRAF* is available upon request. Positive small mutation findings were confirmed by repeat testing in most cases, DNA quantity permitting, for all three methodologies.

Fluorescence In Situ Hybridization (FISH) Analyses

ALK FISH assays were performed with the analyte specific reagent (ASR) LSI *ALK* Dual Color, Break-Apart Rearrangement Probe or the IVD Vysis *ALK* Break Apart FISH Probe Kit (Abbott Molecular). The *MET* FISH assays were performed with laboratory-developed

reagents (DNA insert encompassing *MET* from the RP 11-95I20 BAC clone and a centromere 7 control probe) or with commercial reagents (*MET* (Texas Red) / CEP7 (D7Z1) Green from Cytocell; *MET* (ZyGreen) / CEP7 (ZyOrange) from ZytoVision; Vysis *MET* SpectrumRed / CEP 7 (D7Z1) SpectrumGreen from Abbott Molecular; or Poseidon™ Repeat Free™*c-MET* (7q31) PlatinumBright550 / SE 7 PlatinumBright495 from Kreatech). All probe sets were validated to identify copy number changes in *MET* on 7q31.2 and used a centromere 7 control probe to define the relative copy number alteration.

FISH reagents were validated internally at each of the six diagnostic laboratories that performed testing as part of the LCMC (Supplemental Table 1). Hybridization followed standard procedures and signals were evaluated in at least 50 tumor nuclei per specimen; samples containing fewer than 50 tumor nuclei were considered inadequate. *ALK* rearrangement was defined by split 3'*ALK* - 5'*ALK* signals (separated by a gap ≥2 times the signal diameter) or an isolated 3'*ALK* signal (no 5'*ALK* signal) in ≥15% of tumor cells. [5] *MET* amplification was considered to be present when the *MET* / centromere 7 ratio was > 2.2.

Inter-institutional Assay Validation and Proficiency Testing

The validation cohort included mutant cell lines and de-identified clinical specimens of known genotype collected at one site; extracted DNA was shared with participating laboratories (Supplemental Table 3). Mutation assays for which no corresponding samples suitable for cross-validation between laboratories were available were validated independently by each participating laboratory, using synthetic oligonucleotides harboring the mutation of interest. Control samples for FISH assay validation consisted of a panel of *MET*-amplified and *EML4-ALK* fusion positive lung ACA, provided to each participating laboratory by a single site (Supplemental Table 3).

Proficiency assessment was performed by individual LCMC laboratories on an ongoing basis, in compliance with CLIA guidelines. In addition, we designed a proficiency testing protocol that was carried out at the onset of the study, to serve as extra measure of quality assurance, and to internally document the reliability and accuracy of the tests performed by participating laboratories. One site provided five blinded samples derived from archival tumor containing a minimum of 200 ng of DNA to the other centers and evaluated their mutation results. FISH proficiency assessment consisted of the blinded evaluation of four lung adenocarcinoma surgical cases, two each for *MET* gene amplification and *ALK* gene rearrangement. One unstained 5-micron section and paired hematoxylin and eosin-stained slide per FISH assay was submitted to participating laboratories for analysis.

Confirmation of Cases with Multiple Alterations

Confirmation of FISH results in cases with multiple driver alterations was performed when adequate tissue was available. Confirmation was carried out by repeating the FISH assay and when possible by using an orthogonal technique. ALK immunohistochemistry using clone 5A4 (Abcam, Cambridge, MA) was applied in cases with a mutation and *ALK*-rearrangement, as previously described. [20] *MET* copy number was confirmed using dual color *in situ* hybridization (ISH) probes to the *MET* locus and CEN7 with dual color open

probe software (Ventana Medical Systems, Tucson, AZ). Because dual ISH has been demonstrated to underestimate the probe to centromere ratio as compared to FISH [21], the cutoff for *MET* amplification was defined as a *MET*/centromere 7 ratio of >1.8 for this analysis.

Database systems and Statistical Methods

Data were collected from participating institutions via entry into GeneInsight, a centralized web-based system.

Analyses evaluating efficiency of small-mutation testing were institution-specific, and represented as a ratio of total mutation calls to total number of mutations assessed on the relevant testing platform. Failure to complete all calls occurred due to technical failures plus incomplete testing for various reasons including sample insufficiency.

The Kruskal-Wallis test was utilized for group comparison of continuous variables, and Chi-square test was used for categorical variables.

RESULTS

Subjects Studied and Overall Findings

The frequency of mutations identified in 733 completely tested lung ACA is shown in Figure 1, summarizing the data from our earlier publication on the LCMC experience. [14] Note that in Figure 1, cases with two mutations are represented only once, selecting the mutation that primarily dictated clinical care in each patient. As a result the frequencies of *ALK*, *MET*, and *PIK3CA* alterations appear somewhat lower in this figure. (See Table 4 for details on doubleton mutations.)

When considering the 1007 cases with any genotyping, and including mutations occurring in doubletons, *KRAS* and *EGFR* mutations were seen in 25% and 22% of samples, respectively, and *ALK* rearrangement was detected in 8.5% of cases. Mutations in *ERBB2*, *BRAF*, *PIK3CA*, *NRAS* and *MEK1* as well as *MET* amplification, were all seen at < 3% frequency. *AKT1* was mutated in a single case.

Effects of assay methodology and specimen type on genetic findings

To determine whether assay methodology had an influence on mutation detection, we assessed results according to method of analysis, irrespective of institution. Of 813 specimens with any genotyping performed for which specimen type information was available, 289 were biopsies (36%), 134 were cytology specimens, including effusions and fine needle aspirates (16%), and 390 were surgical resections, including primary tumor resections and resections of metastatic sites (48%). The small mutation completion rate was very high (≥ 98%) for all specimen types (Table 2), which likely reflects the effectiveness of pathology pre-screening to exclude cases with inadequate material. Similarly, there was no significant difference in the frequency of detection of small mutations according to specimen type, with a positive call rate of 55% for biopsies, 56% for cytology specimens, and 56% for surgical resections (p=0.97) (Table 2). *KRAS* and *EGFR* mutations were

somewhat more common in surgical resections and cytology specimens, respectively, but the differences were not statistically significant (Supplemental Table 4).

MET FISH testing was implemented later than other testing at some sites thereby limiting its overall completion rate. Thus, we focused on *ALK* FISH testing to assess FISH completion rates (Table 2). There was no significant difference in *ALK* FISH completion rates based on specimen type, with results returned on 91% of biopsies, 92% of cytology specimens, and 95% of surgical resection specimens (p=0.14). In addition specimen type had no impact on the frequency of *ALK* positive findings (p=0.73, Table 2).

Specimen Sufficiency

Anticipating specimen adequacy for molecular testing and clinical trial enrollment is one of the most challenging aspects of modern clinical oncology. Several sites screened trial candidates for sample sufficiency prior to patient enrollment in the study, thereby reducing the screen fail rate among enrolled subjects. One of the limitations of our data capture strategy emerged at the time of data analysis, when it became clear that site to site variation in pre-screening practices were likely affecting the fail rate at individual institutions. We did not anticipate this issue in advance, and there was no pre-set procedure for pre-screening, and no obligation to capture and document patients who failed to meet study eligibility based on lack of adequate tissue. Therefore, to evaluate the percentage of samples that were not sufficient for these molecular assays, and eliminate site-to-site variation, we focused our analysis on the site with highest mutational testing volume. Of the 470 specimens of known specimen type submitted for testing at this site, 72 (15%) were rejected on the basis of insufficient tumor or being unsuitable for analysis (e.g., decalcification). Surgical resection specimens were significantly less likely to fail pathology screening (5% failure rate) as compared to biopsies and cytology specimens (26% and 35%, respectively, p<0.001, Table 3). We acknowledge that these failure rates may be an underestimate because candidate patients whose tumor specimens were likely to be insufficient at the time of consideration for this trial may not have been enrolled.

Proficiency Testing

A project-specific proficiency testing protocol performed at the beginning of the study evaluated 5 specimens for mutation and 4 specimens for FISH analyses. Eight of nine proficiency samples were correctly scored by the participating laboratories (Supplemental Table 5). One of the mutation samples (DNA 36) yielded unexpected results, with different labs reporting distinct mutations or a technical failure. It is possible that the integrity of this nucleic acid was compromised during processing or shipment, and the sample was classified as an ungraded challenge due to the lack of consensus among testing sites. One site that utilized Sanger sequencing with PNAs was unable to participate because the methodology required more sample material than provided for proficiency testing. For the duration of the study, all sites engaged in proficiency assessment with blinded samples on a regular basis in compliance with CLIA/CAP recommendations.

Analysis of Cases with Multiple Alterations

Twenty-seven cases (2.7% of 1007 samples with any genotyping) had multiple oncogenic driver mutations identified, including 14 with two small mutations and 13 with a small mutation and an *ALK* rearrangement (6) or *MET* amplification (9; two cases had concurrent ALK rearrangement and MET amplification). Of 14 cases with two small mutations, 13 (92%) had a *PIK3CA* mutation in addition to another mutation, including 9 with *EGFR*, 2 with *BRAF*, 1 with *KRAS*, and 1 with *MEK1* mutation. One case had *EGFR* ex19del and *AKT1* c.49G>A (p.E17K) mutations. All of these small mutation findings were validated by repeat analysis at the time of detection.

The 13 cases that had both a small mutation and a positive FISH result are presented in Table 4. *MET* amplification co-occurred nine times with a variety of other alterations including *ALK* rearrangement (2 cases) and *EGFR (2 cases)*, *ERBB2 (1 case)*, and *KRAS* (4 cases) mutations. Replicate FISH testing and/or bright field ISH was performed in 7 of these 9 cases, and all were validated (including 4 by ISH). In the 2 cases with both MET amplification and *ALK* rearrangement, remaining tissue was insufficient for confirmation. *ALK* rearrangement was identified with concurrent small mutations in 4 cases, three with *EGFR* mutations and one with *BRAF* mutation. In two cases, follow-up testing, including using ALK immunohistochemistry and repeat FISH, failed to confirm a functional *ALK* rearrangement (previously reported [21]). In one case, an *EGFR* mutation was confirmed but insufficient tissue remained to confirm the *ALK* rearrangement. This patient has not responded to either EGFR or ALK inhibitor treatment. In another case with confirmed *ALK* and *EGFR* alterations, follow up information was not available.

Clinico-Pathological Associations with Specific Mutations

The prevalence of any small mutation in this cohort was higher in females, those of Asian race, and never-smokers (all $p \leq 0.014$, Figure 2, Supplemental Table 6). There was no association between presence of any mutation and age or stage at diagnosis.

Multiple individual gene mutations showed significant associations with clinicopathologic features (Figure 2, Supplemental Table 6), many of which were expected (6). *EGFR* mutation was significantly associated with female gender, Asian race, never-smoking status, stage IV disease at diagnosis, the presence of bone metastases, and absence of adrenal metastases (all $p < 0.03$). In contrast, *KRAS* mutation correlated with smoking ($p<0.001$), older age at diagnosis ($p<0.001$), lower frequency of bone metastases ($p=0.007$), and white race ($p=0.006$). *ERBB2* mutations were significantly associated with a never-smoker status ($p<0.001$) and Asian race ($p=0.015$). *ALK* rearrangement was associated with a lower age at diagnosis ($p<0.001$), never-smoker status ($p<0.001$) and liver metastases ($p=0.028$). *BRAF* mutations and *MET* amplification were not associated with age, smoking history, stage at diagnosis, race or metastatic pattern.

Approximately one-third of the specimens in the overall cohort were surgical resections. In this subgroup we examined associations between histologic subtype [22] and mutation. (Supplemental Table 7). We found no significant association between predominant histologic subtype and mutation, but there was a trend toward increased *EGFR* mutation

frequency in acinar versus solid-predominant tumors (50/199, 25% vs. 23/165, 14%; p=0.05), those two being the most common subtypes in the surgically-resected subgroup. When considering median percentage of each subtype within a single specimen, none of lepidic, micropapillary, or papillary subtypes showed significant association with EGFR mutation status (Wilcoxon rank-sum test). However, a higher median percentage of acinar subtype within a specimen was associated with EGFR-positive status (median 95% acinar in EGFR-mutant; median 30% acinar in EGFR-wild type; p=0.008), while higher percentage of solid subtype was associated with EGFR-negative status (median 10% solid in EGFR-wild type; median 0% solid in EGFR-mutant; p=0.005).

DISCUSSION

This study demonstrates that collaborative efforts to share protocols and technology between laboratories facilitate comprehensive, multi-institutional tumor mutational profiling. Despite the diversity of analytic approaches employed (Table 1), our results show no appreciable evidence of inter-laboratory or inter-platform variability in mutation detection.

Specimen adequacy is a significant clinical issue in lung cancers since minimally invasive diagnostic procedures are common. In this study, 1,542 patients were enrolled, and only 1,102 (71%) were deemed eligible. The majority of ineligible subjects were due to inadequate pathologic material. Iterative testing often requires recutting/resurfacing (and associated “wasting”) of the tissue block, thus for many patients, tumor cells in the material were exhausted by the time all assays were implemented, and often prior to analysis by routine diagnostic methodology. As a result, only 733 patients had complete genotyping performed, representing 48% of the starting 1,542 subjects enrolled. These observations highlight the challenges of implementing new and labor-intensive molecular testing across multiple institutions and the need for careful planning in advance for tissue optimization. Indeed, our experience during the course of this study led many LCMC institutions to modify their tissue handling and diagnostic workup protocols, including modifying pathology requisitions to flag biopsies for genomic studies, requesting up-front serial tissue sections to reserve for molecular testing, and limiting immunohistochemistry analyses.

Focusing on the single site with the largest testing volume, to eliminate the effect of site-to-site differences in screening approaches, we determined that 35% of cytology specimens and 26% of small biopsies were insufficient as compared to 5% of surgical resections. However, once a specimen was deemed adequate, the sample type did not influence performance, and minor differences between completion rates were felt not to be clinically significant (Table 2). Despite recently-published findings from CAP demonstrating poor inter-laboratory precision in determining tumor percentage based on review of an H&E slide [23], our findings suggest that the pathology prescreen to assess tumor percentage works well as a predictor of success of mutation analyses.

Although the frequencies of mutation seen here (Figure 1) are similar to previous publications, [24-27] they are somewhat higher overall, likely due to referral bias to the tertiary cancer centers making up the LCMC, and provider enrollment of patients who were thought more likely to harbor targetable oncogenic mutation based on clinical and demographic

features. This phenomenon is reflected in the unusually high frequency of women vs. men (60% vs. 40%), and never-smoker and former-smoker vs. current smokers (34%, 59%, and 7%, respectively) in this cohort.

Consistent with prior reports, [28] we found that most oncogenic driver mutations occur in a mutually exclusive fashion. However, we identified 2.7% of cases with two or more putative driver alterations. *PIK3CA* mutations were the mutation type most commonly seen in combination with other alterations. *PIK3CA* mutations are seen in many cancer types, and cause activation of both AKT and mammalian target of rapamycin (mTOR), with myriad downstream effects.[29] The co-occurrence of mutations in *PIK3CA* and *EGFR*, and *PIK3CA* and*KRAS*, in lung adenocarcinoma is well-established.[27] Both *in vitro* studies and analysis of relapse biopsies suggest that *PIK3CA* activating mutations may confer resistance to gefitinib and erlotinib in *EGFR*-mutant lung adenocarcinoma.[29,30] However, the clinical significance of these co-mutations at the time of primary diagnosis remains unclear.

The literature on co-occurrence of *ALK* rearrangement and other oncogenic driver mutations suggests that this is a rare event with unclear implications for therapy. A recently published large series of 1683 lung ACA tested for *EGFR*, *KRAS* and *ALK* demonstrated that these were mutually exclusive alterations. [28] However, other reports suggest that combined *ALK* rearrangement and *EGFR* and/or *KRAS* mutations may occur *de novo* [31,32] and in post-crizotinib treated patients [33], raising the possibility that combined ALK and EGFR blockade may be effective in a rare subset of lung ACA patients. We initially identified four cases with an *ALK* rearrangement combined with another driver mutation. Two proved to be false positives related to apparent poor tissue preservation in one case and an atypical FISH result in another; these false positive cases came to light as a result of negative ALK immunohistochemistry. [20] Since the initiation of the LCMC study, many advances have been made with regard to the implementation of IHC as a screening tool for high ALK expression in NSCLC. Although it was not widely available and hence not widely used during this study, the ALK IHC screening approach has merit. These findings suggest that careful confirmation of dual alterations in *ALK* and another oncogene is warranted to exclude a false positive or non-functional FISH result and to ensure that patients receive proper therapy.

MET amplification is an established mechanism of drug resistance in patients receiving EGFR tyrosine kinase inhibitors, [34] and may be an adverse prognostic indicator in untreated non-small cell lung carcinoma. [35] Our data suggests that *MET* amplification, while rare in untreated specimens, occurs in a variety of genomic contexts. However, the limited number of cases in this study with dual alterations precludes analysis of differential response and outcomes data. The introduction of more comprehensive genomic testing into routine practice should help to elucidate the significance of multiple mutations for therapy selection and patient outcome.

The large number of patient samples evaluated in this study allowed for extensive analysis of mutation and clinicopathologic correlations. Our findings were largely consistent with existing literature, lending support to our results. However, we do acknowledge that many statistical tests were performed, leading the possibility of false positive findings. With this

caveat, our findings reinforce the known associations of *EGFR* mutations with female sex, Asian race and never-smoking status; [26,36] *KRAS* mutation with smoking history; and *ALK* rearrangement with younger age, never-smoking status, and liver metastases. [37] Indeed, nearly half (146 of 335, 44%) of never smokers in this cohort had *EGFR* mutations. However, it is notable that over a third of patients with *EGFR*-mutated or *ALK*-rearranged tumors had some smoking history. Of current smokers, 6% had *EGFR* mutations and 6% had *ALK* rearrangements (Supplemental Table 6). Clear correlations between tumor histologic pattern and mutation type are lacking, driven in part by variable recognition of defined subtypes and by the morphologic complexity of lung adenocarcinoma. After restricting the analysis to the cohort of surgically resected specimens and employing central, expert pathology review, we found *EGFR* mutations more strongly correlated with acinar than with solid subtype histology, although even in this latter group *EGFR* was mutated in 14% of cases. [38,39] These findings thus reinforce the widely adopted recommendations that testing for these targetable alterations be performed on all lung adenocarcinoma patients, irrespective of patient demographics and tumor histologic subtype. [6,40]

This study is one of the largest of its kind to address the many challenges that arise in the course of molecular testing of solid tumors for therapeutic decision-making. It is reassuring that geographically diverse institutions serving a wide range of patient populations generate similar results using a variety of sample types and testing platforms, underscoring the ability of advanced molecular diagnostic laboratories to establish, validate and implement high-fidelity assays. However, our experience clearly highlights the drawbacks of sequential targeted genotyping in practice. Menu variation in multiplex genotyping assays means that individual institutions may offer incomplete coverage at certain targets, whereas Sanger sequencing provides more comprehensive coverage but at much lower analytic sensitivity and requires significantly more input DNA. FISH studies drive an increased need for tumor tissue, presenting a particular problem in lung adenocarcinoma where the number of gene fusions and copy number targets continues to grow. As a result, this iterative approach to mutational profiling appears unsustainable in the face of increasing numbers of targetable alterations, and new approaches to testing are needed and are indeed in various stages of implementation nationally, such as next generation sequencing (NGS).

In summary, The LCMC demonstrates the feasibility of widespread implementation of mutational profiling in the clinical care of lung cancer patients. It lays the groundwork for future collaborative efforts that will be necessary to fully characterize the mutational spectrum of lung cancers in light of the relative rarity of many of the newly recognized driver alterations in this tumor type. As clinical oncology and molecular diagnostics embrace new technological platforms, this type of collaborative study is critical for validation of biomarkers and for facilitating identification of candidates for clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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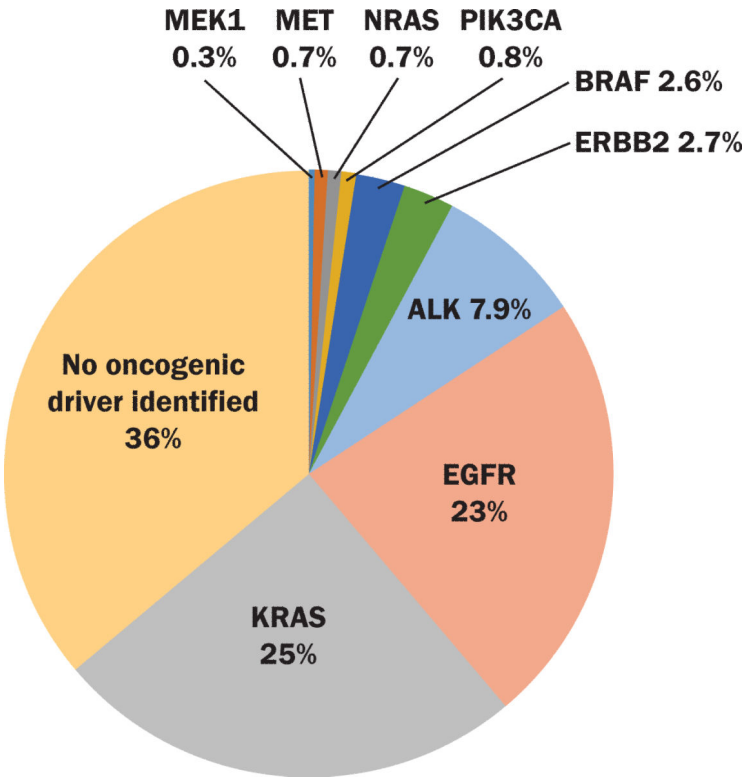

Figure 1. Mutations identified in the LCMC cohort

A pie chart is shown in which the size of each slice is proportional to the mutation frequency in the full genotyping set of 733 patients. Cases with two mutations are represented only once based on the mutation that primarily dictated clinical care in each patient. As a result the frequencies of *ALK*, *MET*, and *PIK3CA* alterations appear somewhat lower in this figure. (See Table 4 for details.) Frequencies presented here also differ slightly for some genes in comparison to frequencies for the any-genotyping group (n=1007). Please see details of the full-genotyping vs. any-genotyping cohorts, and a comprehensive breakdown of all mutations identified in our original paper on the LCMC experience, ref. 14).

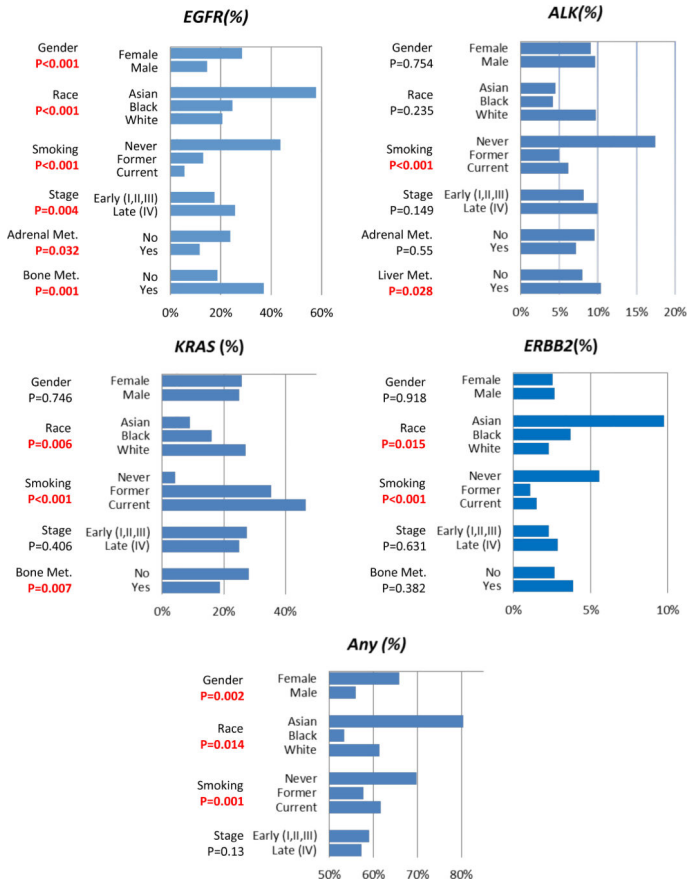

Figure 2. Demographic and prognostic associations with any and individual specific mutations

Statistically significant and other notable associations are shown in the form of bar graphs for several mutation types. “Any” refers to any identified mutation. KRAS, EGFR, and ERBB2 consist of all point mutations occurring in those genes; ALK refers to translocations involving ALK, and MET refers to amplification of the MET locus, identified by FISH. More detailed demographic and prognostic association data are provided in Supplemental Table 7.

Table 1

Analytic methods and sensitivity across six major testing sites.

Number of sites	Platform	Analytic sensitivity	Minimum accepted tumor cellularity†
4	SNapShot	5-10%	10-20%*
	PCR-sizing	5-10%	10-20%
1	Sequenom	5-10%	25%
	PCR-sizing	5-10%	25%
1	Sanger	25%	50%
	Sanger with PNA*	5%	10%

These six sites were responsible for more than 90% of mutation testing. A single site (not included here) produced only 1% of the overall results and utilized a different methodology (pyrosequencing) and thus is not included in this cross-platform analyses.

*Peptide nucleic acid (PNA) clamps were used to enhance sensitivity of mutation detection in *EGFR*, *KRAS* and *BRAF* genes.

†Manual microdissection to isolate tumor-rich areas for DNA extraction was performed at all sites.

Table 2

Genotyping efficiency and positive call rate per specimen type.

Subsets	Biopsy	Cytology	Surgical	p-value
Specimens tested at all LCMC sites				
Any small genotyping & known specimen type (n=813)	*n=289*	*n=134*	*n=390*	
Small-mutation genotyping efficiency (mean call rate across cases)	98%	99%	98%	0.04
Small-mutation positive call rate (proportion of cases detected with any small mutation)	55% (159)	56% (75)	56% (218)	0.97
Any genotyping & known specimen type (n=855)	*n=306*	*n=145*	*n=404*	
ALK-FISH genotyping efficiency (proportion of cases with successful *ALK* testing)	91% (279)	92% (133)	95% (383)	0.14
***ALK-FISH* genotyping & known specimen type (n=795)**	*n=279*	*n=133*	*n=383*	
ALK-FISH positive call rate (proportion of cases detected with *ALK* rearrangement)	10% (27)	8% (10)	8% (32)	0.73
Specimens tested at single LCMC site with plurality of testing				
Any small genotyping & known specimen type (n=360)	*n=85*	*n=33*	*n=242*	
Small-mutation genotyping efficiency (mean call rate across cases)	96%	100%	99%	0.49
Small-mutation positive call rate (proportion of cases detected with any small mutation)	53% (45)	45% (15)	56% (136)	0.48
Any genotyping & known specimen type (n=398)	*n=100*	*n=43*	*n=255*	
ALK-FISH genotyping efficiency (proportion of cases with successful *ALK* testing)	100% (100)	93% (40)	96% (245)	0.06
***ALK*-FISH genotyping & known specimen type (n=385)**	*n=100*	*n=40*	*n=245*	
ALK-FISH positive call rate (proportion of cases detected with *ALK* rearrangement)	13% (13)	15% (6)	8% (20)	0.22

Specimens of unspecified type were not included in this analysis.

Table 3

Specimen sufficiency according to type of specimen, in 470 specimens of known type, tested at a single LCMC site with plurality of testing.

Screen failure for insufficient material	Biopsy (n=136)	Cytology (n=66)	Surgical (n=268)	p-value
No	74% (100)	65% (43)	95% (255)	<0.001
Yes	26% (36)	35% (23)	5% (13)	

Table 4

Doubleton molecular alterations: Dual FISH or FISH-mutation.

Doublet on case #	Alteration #1	Alteration #2	Confirmed, same technique	Confirmed, orthologous technique	Comments
1	*ALK*	*MET*	ND	ND	Insufficient tissue remaining
2	*ALK*	*MET*	ND	ND	Insufficient tissue remaining
3	*ALK*	*EGFR* c.2582T>A	*ALK*- ND *EGFR*-Yes	ND	Patient experienced minimal response to sequential erlotinib and crizotinib therapies
4	*ALK*	*EGFR* c.2573T>G	*ALK*- No *EGFR*-Yes	No	ALK IHC negative. Repeat *ALK* FISH showed atypical rearrangement, considered negative. Patient responded to erlotinib[21]
5	*ALK*	*EGFR* ex19del	Yes	ND	No follow up data available
6	*ALK*	*BRAF* c.1799T>A	ALK-No *BRAF*-Yes	No	ALK IHC negative. Repeat *ALK* FISH negative. Patient failed to respond to crizotinib [21]
7	*EGFR* ex19del	*MET*	Yes	ND	*MET* dual ISH was a technical failure
8	*EGFR* c.2573T>G	*MET*	Yes	ND	Insufficient tissue available for orthogonal testing
9	HER2 ex20ins	*MET*	Yes	Yes	*MET* dual ISH positive
10	*KRAS* c.35G>C	*MET*	Yes	Yes	*MET* dual ISH positive
11	*KRAS* c.35G>T	*MET*	Yes	ND	No tissue available for orthogonal testing
12	*KRAS* c.34G>T	*MET*	Yes	Yes	*MET* dual ISH positive
13	*KRAS* c.34G>T	*MET*	Yes	Yes	*MET* dual ISH positive

ND, not done.